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### Citation for published version:

Foster, K, Sheridan, J, Veiga-Fernandes, H, Roderick, K, Pachnis, V, Adams, R, Blackburn, C, Kioussis, D & Coles, M 2008, 'Contribution of neural crest-derived cells in the embryonic and adult thymus', *The Journal of Immunology*, vol. 180, no. 5, pp. 3183-3189.

### Link:

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### Document Version:

Publisher's PDF, also known as Version of record

### Published In:

The Journal of Immunology

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*J Immunol* 2008; 180:3183-3189; ;  
<http://www.jimmunol.org/content/180/5/3183>

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<b>Supplementary Material</b>	<a href="http://www.jimmunol.org/content/suppl/2008/02/27/180.5.3183.DC1.html">http://www.jimmunol.org/content/suppl/2008/02/27/180.5.3183.DC1.html</a>
<b>References</b>	This article <b>cites 34 articles</b> , 19 of which you can access for free at: <a href="http://www.jimmunol.org/content/180/5/3183.full#ref-list-1">http://www.jimmunol.org/content/180/5/3183.full#ref-list-1</a>
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# Contribution of Neural Crest-Derived Cells in the Embryonic and Adult Thymus<sup>1</sup>

Katie Foster,\* Julie Sheridan,<sup>‡</sup> Henrique Veiga-Fernandes,\* Kathleen Roderick,\* Vassilis Pachnis,<sup>†</sup> Ralf Adams,<sup>¶</sup> Clare Blackburn,<sup>‡</sup> Dimitris Kioussis,<sup>2\*</sup> and Mark Coles<sup>2\*§</sup>

Neural crest (NC)-derived mesenchyme has previously been shown to play an important role in the development of fetal thymus. Using *Wnt1-Cre* and *Sox10-Cre* mice crossed to *Rosa26<sup>eYFP</sup>* reporter mice, we have revealed NC-derived mesenchymal cells in the adult murine thymus. We report that NC-derived cells infiltrate the thymus before day 13.5 of embryonic development (E13.5) and differentiate into cells with characteristics of smooth muscle cells associated with large vessels, and pericytes associated with capillaries. In the adult organ at 3 mo of age, these NC-derived perivascular cells continue to be associated with the vasculature, providing structural support to the blood vessels and possibly regulating endothelial cell function. *The Journal of Immunology*, 2008, 180: 3183–3189.

**T**he thymus rudiment is first colonized by lymphocyte progenitor cells between days 10.5 and 12.5 of embryonic development (E10.5–E12.5) (1). In the thymic context, hemopoietic progenitors undergo complex selection and maturation processes resulting in the generation of a diverse T cell repertoire essential for the establishment of protective immunity. In addition to the hemopoietic cells, the thymic parenchyma is also comprised of blood vessels, connective tissue, and epithelial cells, which form a highly specialized microenvironment (2).

In the early stages of thymic organogenesis (E10.5–E14.5), lymphocyte progenitors migrate into the thymic rudiment through the perithymic mesenchyme in response to chemoattractant factors (3). After blood vasculature development, direct lymphocyte trafficking between the blood circulation and the thymus becomes the main mode of thymus colonization.

It is thought that endothelial cells from vessels surrounding the thymus proliferate and migrate into the organ toward angiogenic stimuli, such as vascular endothelial growth factor (VEGF)<sup>3</sup> (4).

VEGF, produced by thymic epithelial cells and mesenchymal cells (5), is an endothelial cell mitogen and a permeability-enhancing factor that stimulates angiogenesis in response to hypoxia and increased glucose concentration or pH changes (6). VEGF receptor-positive endothelial cells upon binding VEGF are induced to proliferate, resulting in formation of sprouting processes or immature blood vessel structures. Recently, it has been demonstrated that pericytes are an important source of VEGF and may migrate ahead of endothelial cells guiding the sprouting processes (7). Endothelial cells produce platelet-derived growth factor (PDGF)-B, which in turn attracts PDGFR- $\beta^+$  perivascular cells to the walls of growing blood vessels (8). Perivascular cells, such as pericytes and smooth muscle cells, wrap around the vessels providing structural support and regulating endothelial cell function. In the absence of perivascular cells or PDGFR- $\beta$  signaling, vessel structures are abnormal and prone to hemorrhage (9). Indeed, this is a characteristic of pathological states such as edema and diabetic retinopathy, where vessels appear abnormal and fragile (10).

Neural crest cells (NCCs) have been shown to play a major role in the formation of the thymus rudiment. NCCs migrate extensively from the neural folds and contribute to various cell populations in different tissues throughout the embryo (11–13). Interestingly, NCCs were shown to contribute also to connective tissues in the thymic capsule and to surround blood vessels inside the thymus of chick-quail chimeras (14). Between E9.5 and E12.5 the development of the murine thymus is dependent on interactions between epithelial and neural crest (NC)-derived cells. Thus, NC-derived mesenchyme stimulates proliferation and possibly also maturation of epithelial cells, which upon differentiation express among others stem cell factor, delta-like 4, and MHC class II molecules required for T cell development (15).

The functional importance of NC-derived cells during thymus organogenesis has been further suggested by experiments in which their removal or reduction in the pharyngeal region results in greatly reduced size or complete absence of the organ (11). Notably, normal expansion of the epithelial compartment does not occur if NC-derived cells are absent (16). However, it has been reported, based on tissue-specific heritable genetic labeling, that once epithelial cells have fully gained the competence to support lymphocyte differentiation, and the vasculature of the thymus is complete, the contribution of NCCs in the thymus is greatly reduced or absent (17, 18). Thus, there is controversy regarding the role of NCCs beyond the early stages of organogenesis.

\*Molecular Immunology and <sup>†</sup>Developmental Neurobiology, National Institute for Medical Research; <sup>‡</sup>Centre Development in Stem Cell Biology, Institute for Stem Cell Research, School of Biological Sciences University of Edinburgh, Edinburgh; and <sup>§</sup>Immunology and Infection Unit, Department of Biology, University of York and The Hull York Medical School, York, United Kingdom; and <sup>¶</sup>Max-Planck-Institute for Molecular Biomedicine, Department of Tissue Morphogenesis, and University of Münster, Faculty of Medicine, Münster, Germany

Received for publication September 13, 2007. Accepted for publication January 2, 2008.

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<sup>1</sup> This work was supported by the Medical Research Council. H.V.-F. and K.F. are supported by a grant from the European Union (LSHG-CT-2003-503259). R.A. is supported by Cancer Research U.K. C.B. and J.S. are supported by the Leukemia Research Fund, the European Union, and the Medical Research Council.

<sup>2</sup> Address correspondence and reprint requests to Dr. Dimitris Kioussis, Division of Molecular Immunology, National Institute for Medical Research, Mill Hill, London, NW7 1AA, U.K. E-mail address: dkiouss@nimr.mrc.ac.uk or Dr. Mark Coles, Immunology and Infection Unit Department of Biology, University of York and The Hull York Medical School, P.O. Box 373, York, YO10 5YW, U.K. E-mail address: mc542@york.ac.uk

<sup>3</sup> Abbreviations used in this paper: VEGF, vascular endothelial growth factor;  $\alpha$ -SMA,  $\alpha$ -smooth muscle actin; BABB, benzyl alcohol and benzyl benzoate; E10.5–E18.5, days 10.5–18.5 of embryonic development; eYFP, enhanced yellow fluorescent protein; NCC, neural crest cell; NC, neural crest; PDGF, platelet-derived growth factor.

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To dissect the contribution of NC-derived cells in thymus development, we have used a tissue-specific heritable genetic labeling system. Thus, Cre recombinase was expressed under the control of either the *Wnt1* (19) or *Sox10* (20) promoter and regulatory elements, and Cre activity was reported via activation of a “silent” enhanced yellow fluorescent protein (eYFP), in which the *eYfp* gene preceded by a triple polyadenylation signal flanked by two loxP sites is knocked into the *Rosa26* locus (21). As *Wnt1* and *Sox10* are expressed in the neural plate where NCCs originate (13, 20, 22), the latter cells will express Cre, thus deleting the transcriptional stop in front of the *eYfp* and indelibly marking these cells and their progeny by eYFP expression. We herein show that NC-derived cells that associate mainly with the thymic capsule at E12.5 enter the parenchyma before E13.5, remain there throughout gestation, and continue to be a major cellular component in the adult organ at 3 mo of age. Furthermore, we demonstrate that NC-derived cells differentiate into pericytes and smooth muscle cells, which are two different cell subsets that form a supportive network for thymic blood vessels. Our results argue for a role of NC-derived cells that extends beyond the early stages of thymus organogenesis.

## Materials and Methods

### Mice

*Wnt1-Cre* (19), *Sox10-Cre* (20), *Rosa26<sup>eYfp</sup>* (21), and C57BL/6 mice were bred at the animal facilities in the Medical Research Council–National Institute of Medical Research (U.K.). Embryos were generated by crossing *Wnt1-Cre; Rosa26<sup>eYfp</sup>* mice with C57BL/6 females. All mice were bred and kept under specific pathogen-free conditions. Experiments were conducted according to the U.K. Home Office rules for animal experimentation.

### Stereo microscopy

eYFP expression was analyzed using a Zeiss M2Bio (Carl Zeiss) stereo-fluorescent microscope. Images were acquired with an ORCA-ER (Hamamatsu) camera and Openlab software (Improvision). eYFP expression was analyzed using a wide-band GFP filter cube (470/500LP) (Kramer Scientific) with green (525) and red (630) filters (Chroma Technology). Images were merged in Openlab and contrast-enhanced in Photoshop (Adobe).

### Whole-mount immunohistochemistry

Embryos and organs were fixed in 4% paraformaldehyde at 4°C and washed in 0.15% PBS-Triton-X100. Sections (100  $\mu$ m) were made after embedding the fixed organs in 8% agarose and using a vibratome (Leica Microsystems). Tissues were washed and blocked in 10% serum and subsequently stained with Abs against eYFP directly conjugated to Alexa Fluor 647 or 594 (Invitrogen: Molecular Probes), endomucin (kind gift from Prof. D. Vestweber), desmin (Sigma-Aldrich),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)-Cy3 (Abcam), PDGFR- $\alpha$  (R&D Systems), PDGFR- $\beta$  (R&D Systems), BrdU (Abcam), and with appropriate secondary Abs against rat IgG (Alexa Fluor 594), mouse IgG (Alexa Fluor 594), and rabbit IgG (Alexa Fluor 594). Embryos negative for Cre resulting from *Wnt1-Cre; Rosa26<sup>eYfp</sup>*  $\times$  C57BL/6 breedings were stained with Abs detecting eYFP and used as negative controls. Background autofluorescence was collected by excitation with 488-nm laser and emission collected between 490 and 515 nm.

### Frozen section preparation and staining

Tissues were embedded in OCT compound (Tissue-Tek), snap frozen on dry ice, and stored at  $-80^{\circ}\text{C}$ . Sections were cut using a cryostat (Leica CM1900) at 10- $\mu$ m thickness and collected onto poly-L-lysine-coated glass slides (VWR International). Sections were air dried for 20 min before being fixed for 2 min in 100% acetone ( $-20^{\circ}\text{C}$ ) and air dried for a further 20 min. Sections were blocked in 5% serum and subsequently stained with Abs against CD31 (BD Pharmingen) and PanK (Dako) and detected with appropriate secondary Abs: anti-rat IgG (Alexa Fluor 488 and anti-mouse IgG (Alexa Fluor 568). Slides were rinsed with water and air dried before mounting with VECTASHIELD HardSet mounting medium (Vector Laboratories).

### Confocal microscopy

For detection of immunofluorescence, samples were analyzed using a Leica SP2 confocal or Leica AOBs confocal microscope (Leica Microsystems). Tissue stained in whole-mount was optically cleared using benzyl alcohol and benzyl benzoate (BABB) (Sigma-Aldrich) before imaging. Confocal images are presented as single sections, as a merge of a number of serial sections, or as a three-dimensional rendering of many serial sections. Three-dimensional renderings were generated using Volocity software (Improvision).

### Flow cytometric cell sorting

NCCs were analyzed by digesting the thymus with collagenase D (Roche) for 1 h at  $37^{\circ}\text{C}$ . The resulting single-cell suspension containing eYFP-expressing cells was stained with directly conjugated Abs against CD45 (eBioscience), MHC II (BD Biosciences), CD31 (eBioscience), and  $\alpha$ -SMA; and mouse monoclonal anti-NG2 (Sigma-Aldrich) revealed with anti-mouse PE (eBioscience), biotinylated anti-mouse PDGFR- $\alpha$  (R&D Systems), and biotinylated anti-mouse PDGFR- $\beta$  (R&D Systems) both revealed with APC- or PE-conjugated streptavidin. Before  $\alpha$ -SMA staining, cells were fixed in 2% paraformaldehyde and permeabilized with 90% methanol. Samples were acquired on a FACSCalibur (BD Biosciences) and analyzed in FlowJo (TreeStar).

### BrdU administration

Pregnant female mice were given one i.p. injection of BrdU (66  $\mu\text{g/g}$  of mouse) at E13.5, E14.5, or E15.5. Embryos were recovered 2 days later and microdissected to remove the thymic rudiment. After fixation (4% paraformaldehyde), thymi were incubated with hydrochloric acid, washed with borate buffer before staining with an Ab recognizing BrdU, and analyzed by confocal microscopy.

## Results

### NC-derived cells in fetal and adult mice

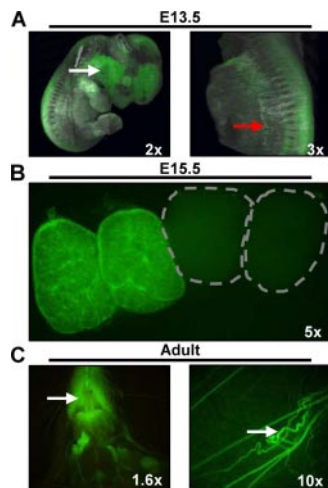
To determine the pattern of eYFP expression in *Wnt1-Cre; Rosa26<sup>eYfp</sup>* reporter mice, we have analyzed both embryonic and 3-mo-old mice by stereo and confocal microscopy. At E13.5, we show that eYFP is expressed in the craniofacial region (Fig. 1A, *left*) and in the neurons in the trunk of the embryo (Fig. 1A, *left and right*). In the thoracic cavity, eYFP expression is detected on the aorta and other arteries derived from the pharyngeal arches (not shown), as well as on the thymic rudiment (Fig. 1B). In the 3-mo-old mouse, eYFP-expressing cells are seen in craniofacial bones and tissues (Fig. 1C, *left*), as well as in peripheral nerves (Fig. 1C, *right*). Similar patterns of expression were observed in another reporter mouse in which eYFP is also expressed by NC-derived cells following Cre expression under the control of NC-specific promoter from the *Sox10* gene (data not shown). This analysis showed that eYFP expression in tissues and organs from embryonic and 3-mo-old *Wnt1-Cre; Rosa26<sup>eYfp</sup>* mice is restricted to NC-derived tissues, as previously described (18, 19, 23).

### NC-derived cells in the fetal thymus

To follow the kinetics of colonization of the thymic rudiment by NC-derived cells, we examined the pattern of eYFP expression in E13.5, E15.5, and E17.5 embryos and newborn mice. At E13.5, some eYFP<sup>+</sup> cells are present inside the thymus, but most are located at the capsule (Fig. 2A, *left*). At E15.5, eYFP<sup>+</sup> cells are again found mainly at the capsule, but their frequency is increased inside the thymic rudiment, where they form trabeculae that appear to extend from the capsule into the parenchyma (Fig. 2A, *middle*). At E17.5, eYFP<sup>+</sup> cells are still found at the periphery of the thymus, while those within the organ have organized into a three-dimensional network (Fig. 2A, *right*; Supplemental movie<sup>4</sup>). Examination of sections from newborn *Wnt1-Cre; Rosa26<sup>eYfp</sup>* thymi showed that eYFP<sup>+</sup> cells are still present within the organ, with many associated with the capsule (Fig. 2B). No eYFP<sup>+</sup> cells were

<sup>4</sup> The online version of this article contains supplemental material.





**FIGURE 1.** Expression of eYFP in fetal and adult *Wnt1-Cre;Rosa26<sup>eYFP</sup>* mice. *A*, Whole-mount E13.5 *Wnt1-Cre;Rosa26<sup>eYFP</sup>* embryos were fixed, stained with an Ab recognizing eYFP, optically cleared with BABB, and analyzed by confocal microscopy. Serial optical sections of the whole embryo were captured and reconstituted to form a three-dimensional rendering. White arrow points to craniofacial structures; red arrow, trunk neurons. *B*, E15.5 thymi from *Wnt1-Cre;Rosa26<sup>eYFP</sup>* (left) and wild-type littermate control thymi (right) were microdissected and subsequently analyzed by stereo microscopy. Dotted gray line indicates outlines of wild-type thymus lobes. *C*, left, Location of eYFP<sup>+</sup> cells in the craniofacial region was determined in 3-mo-old *Wnt1-Cre;Rosa26<sup>eYFP</sup>* mice by stereo microscopy. White arrow points to facial region. Right, Mice were further dissected to reveal expression of eYFP on peripheral nerves. White arrow points to axons.

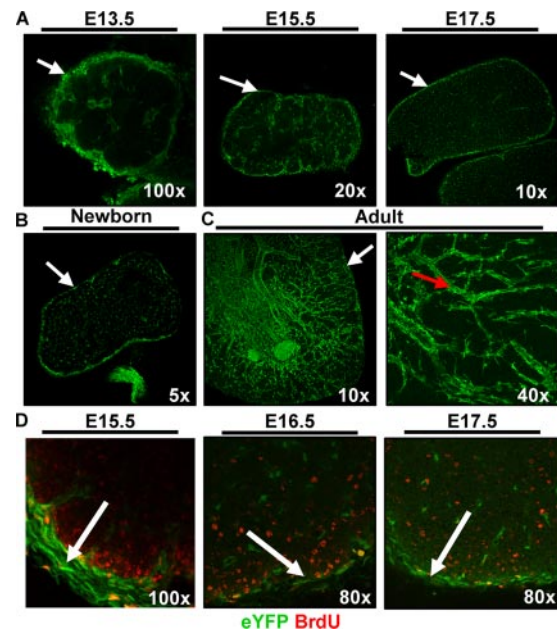
detected in the embryonic or newborn thymus of nontransgenic mice (data not shown). Thus, we conclude that NC-derived cells enter the thymic rudiment before E13.5, remain there throughout gestation, and are still present in the newborn thymus.

To determine whether cells derived from the NC persist in the thymus beyond birth, we analyzed the distribution of eYFP<sup>+</sup> cells in 3-mo-old *Wnt1-Cre;Rosa26<sup>eYFP</sup>* mice. eYFP-expressing cells are readily detectable in the adult thymus at this age, forming a three-dimensional network (Fig. 2*C*, left), with fewer cells being found at the capsule than at embryonic stages. High-magnification confocal analysis of thymus sections from 3-mo-old mice showed that most cells are associated with structures reminiscent of vascular networks (Fig. 2*C*, right). Measurement of the diameter of vessel-like structures shows that cells expressing eYFP are found in association with vessels ranging from 4.5 to 30  $\mu$ m in diameter, indicating a contribution to both capillaries and larger vessels. This finding suggested that NC-derived cells in the adult thymus at 3 mo of age are intimately associated with the blood vasculature.

To determine whether proliferation contributes to thymic colonization by NCCs during organogenesis, *Wnt1-Cre;Rosa26<sup>eYFP</sup>* pregnant females were injected once with BrdU at E13.5, E14.5, or E15.5, and the embryos were recovered 2 days later. We found that the vast majority of eYFP<sup>+</sup> cells were BrdU-negative when compared with the extensive incorporation seen in hemopoietic cells. This suggested that the influx and increase of eYFP<sup>+</sup> cells in the thymus occur mainly by NCC redistribution within the thymus (Fig. 2*D*).

*NCCs are present within the organ and differentiate into perivascular cells before E13.5*

NC-derived mesenchymal cells have been shown to contribute to connective tissues surrounding the thymic blood vasculature in the

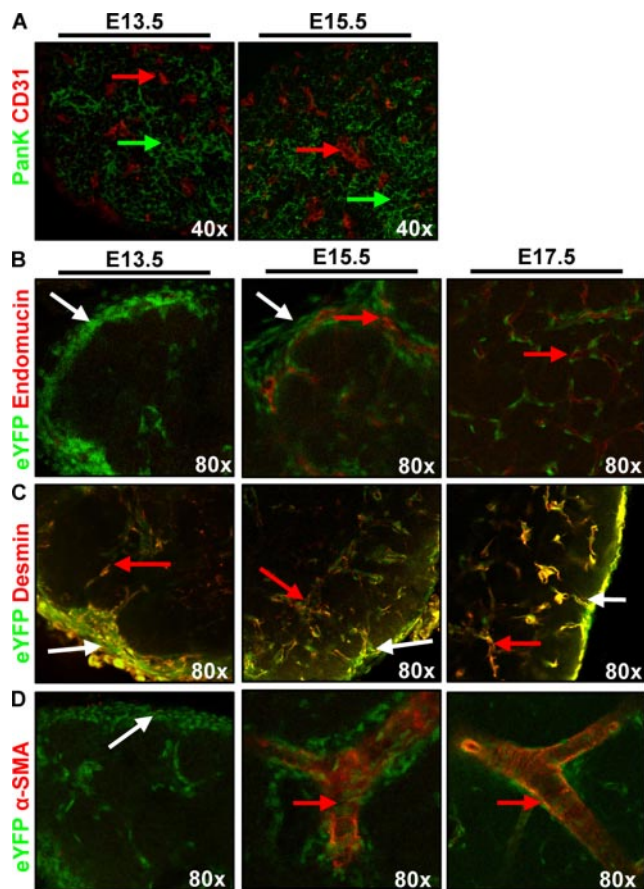


**FIGURE 2.** Confocal analysis of eYFP expression in fetal and adult thymus from *Wnt1-Cre;Rosa26<sup>eYFP</sup>* mice. *A*, Thymi were dissected from *Wnt1-Cre;Rosa26<sup>eYFP</sup>* at E13.5 (left), E15.5 (middle), and E17.5 (right), fixed in 4% paraformaldehyde and stained with an anti-eYFP Ab, optically cleared with BABB, and analyzed by confocal microscopy. *B*, Thymi from newborn *Wnt1-Cre;Rosa26<sup>eYFP</sup>* mice were dissected, fixed and cut in 100- $\mu$ m sections, stained with an anti-eYFP Ab, and analyzed by confocal microscopy. *C*, Thymi from *Wnt1-Cre;Rosa26<sup>eYFP</sup>* mice at 3 mo of age were dissected, processed as previously described, stained with an anti-eYFP Ab, and analyzed by confocal microscopy. White arrows point to capsule of the thymic lobe; red arrow, vessel structures. *D*, E15.5, E16.5, and E17.5 *Wnt1-Cre;Rosa26<sup>eYFP</sup>* embryos were taken from pregnant female mice that had been injected with one dose of BrdU 2 days earlier (66  $\mu$ g/g of mouse). Thymic rudiments were dissected from the embryos, processed as previously described, and stained with Abs against BrdU (red) and eYFP (green). Sections were optically cleared with BABB and analyzed by confocal microscopy. White arrows point to capsule of thymic lobe.

chicken embryo (14). However, their precise function in the murine thymus remains controversial. To assess the kinetics of NC-derived cell contribution to connective tissue components such as perivascular cells, sections of thymic tissue at different stages of embryogenesis were stained with Abs recognizing markers specific for endothelial cells (CD31 and endomucin), pericytes (desmin), and smooth muscle cells ( $\alpha$ -SMA). At E13.5, we found that CD31<sup>+</sup> cells are already present throughout the thymic rudiment, indicating that endothelial cells, or endothelial precursor cells, are already present at this stage (Fig. 3*A*). Endomucin, thought to be expressed on mature endothelial cells (24), is not expressed until E15.5 (Fig. 3*B*). As mentioned above, a network of NC-derived cells is established in the organ by E13.5, and at this stage, eYFP<sup>+</sup> cells express the pericyte marker, desmin (Fig. 3*C*). In contrast,  $\alpha$ -SMA expression is not detected until after E15.5 when the formation of the blood vessel network is complete. From this stage onward eYFP<sup>+</sup> cells also start expressing  $\alpha$ -SMA (Fig. 3*D*).

#### *NC-derived cells in the thymus express PDGF receptors*

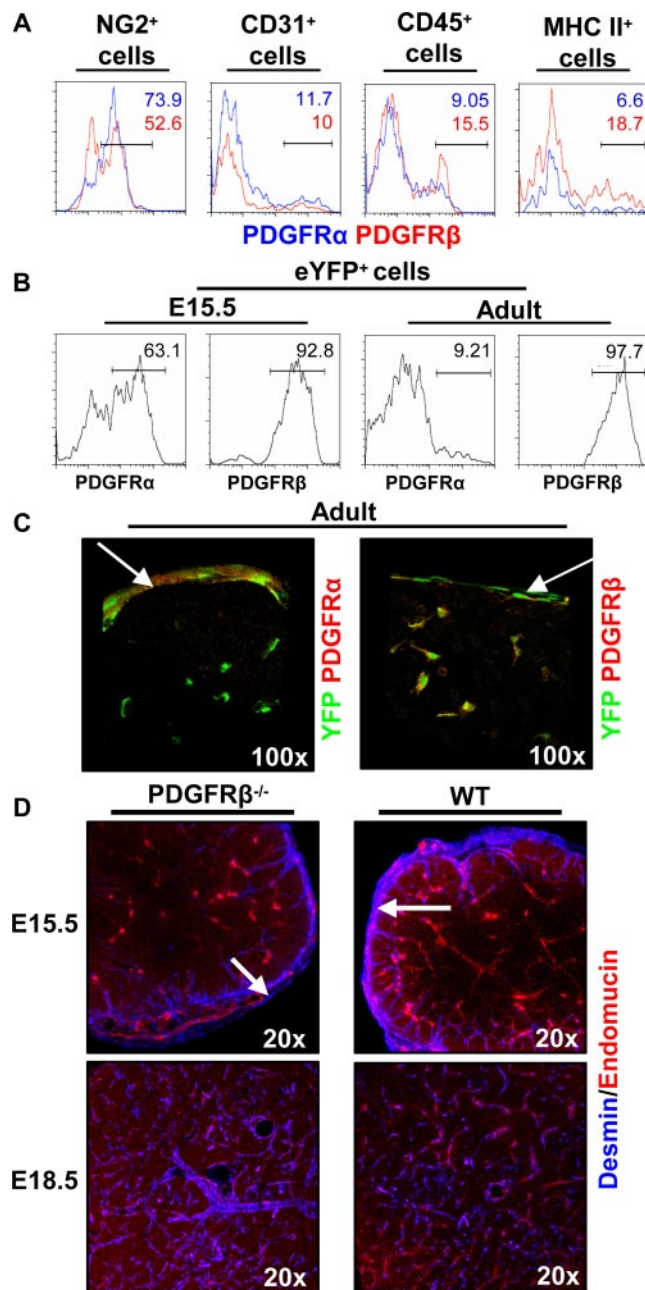
Cells with mesenchymal properties in the embryonic thymus have been reported to derive from NCCs (this report and Refs. 23, 25). PDGF receptor expression is characteristic of mesenchymal cells, and binding of PDGF mitogens regulates a number of processes including gene regulation and cell cycle control. To determine



**FIGURE 3.** Development of blood vessel and eYFP<sup>+</sup> networks in embryonic thymus. **A**, Thymi from C57BL/6 embryos were dissected at E13.5 (left) and E15.5 (right) and stained with Abs recognizing pankheratin (green) and CD31 (red). Red arrows point to endothelial precursors; green arrows, epithelial cells. **B–D**, Thymi from *Wnt1-Cre;Rosa26<sup>eYfp</sup>* embryos were dissected at E13.5 (left column), E15.5 (middle column), and E17.5 (right column), processed as previously described, and stained with Abs recognizing eYFP (green), endomucin (red) (**B**), desmin (red) (**C**), and  $\alpha$ -SMA (red) (**D**), and analyzed by confocal microscopy. White arrows point to capsule of thymic lobe; red arrows, vessels.

which cellular compartments express PDGF receptors in the thymus, we analyzed the expression of PDGFR- $\alpha$  and PDGFR- $\beta$  receptors on perivascular (NG2), endothelial (CD31), hemopoietic (CD45), and epithelial cell populations (MHC class II) in thymi from 3-mo-old C56BL/6 mice. We show that NG2 is expressed by 73.9 and 52.6%, CD31 by 11.7 and 10%, CD45 by 9.05 and 15.5%, and MHC class II by 6.6 and 18.7% of PDGFR- $\alpha$ <sup>+</sup> and PDGFR- $\beta$ <sup>+</sup> populations, respectively (Fig. 4A). Thus, PDGFR- $\alpha$  and PDGFR- $\beta$  are preferentially expressed by cells of a mesenchymal nature, including pericytes.

To determine whether NCCs are also involved in the PDGFR/PDGF axis of signaling during thymic development, we studied the expression of PDGFR- $\alpha$  and PDGFR- $\beta$  in thymic eYFP<sup>+</sup> cells, from *Wnt1-Cre;Rosa26<sup>eYfp</sup>* mice, by flow cytometry. In one such analysis of E15.5 thymic rudiments, 63.1% of eYFP<sup>+</sup> cells express PDGFR- $\alpha$  and 92.8% express PDGFR- $\beta$  (Fig. 4B, left two panels). Interestingly, in the 3-mo-old thymus the majority (97.7%) of eYFP<sup>+</sup> cells still express PDGFR- $\beta$ , but PDGFR- $\alpha$  was found only on 9.2% of the cells (Fig. 4B, right two panels). This was confirmed by confocal microscopy, which showed that PDGFR- $\alpha$  is expressed only by eYFP<sup>+</sup> cells found in the capsule of the 3-mo-old thymus, whereas PDGFR- $\beta$  was expressed by eYFP<sup>+</sup> cells found both at the capsule and within the organ (Fig. 4C).



**FIGURE 4.** eYFP-expressing cells in the adult thymus express mesenchymal markers PDGFR- $\alpha$  and PDGFR- $\beta$ . **A**, Thymi from 3-mo-old C57BL/6 mice were digested with collagenase, stained with markers recognizing perivascular cells (NG2), endothelial cells (CD31), hemopoietic (CD45, CD4, and CD8), and epithelial cells (MHC class II), and analyzed by flow cytometry. CD4<sup>+</sup> and CD8<sup>+</sup> cells were gated out of the analysis, and the percentages of NG2<sup>+</sup>, CD31<sup>+</sup>, CD45<sup>+</sup>, and MHC class II<sup>+</sup> cells expressing PDGFR- $\alpha$  (blue line) and PDGFR- $\beta$  (red line) were calculated from the remaining CD4<sup>+</sup>CD8<sup>+</sup> cells. **B**, Thymi from E15.5 *Wnt1-Cre;Rosa26<sup>eYfp</sup>* embryos and 3-mo-old mice were digested with collagenase and analyzed by flow cytometry for expression of PDGFR- $\alpha$  and PDGFR- $\beta$  on the eYFP<sup>+</sup> population. **C**, Sections (100  $\mu$ m) were cut from thymi of *Wnt1-Cre;Rosa26<sup>eYfp</sup>* 3-mo-old mice and processed as previously described. Sections were stained with Abs against eYFP (green), PDGFR- $\alpha$  (red) (**B**, left), and PDGFR- $\beta$  (red) (**B**, right) and analyzed by confocal microscopy. White arrows point to capsule of the thymus. **D**, Thymi from E15.5 (top row) and E18.5 (second row) PDGFR- $\beta$ <sup>-/-</sup> (left) and wild-type littermate control embryos (right) were dissected, processed as previously described, and stained with Abs recognizing desmin (blue) and endomucin (red). White arrows point to capsule of the thymus.



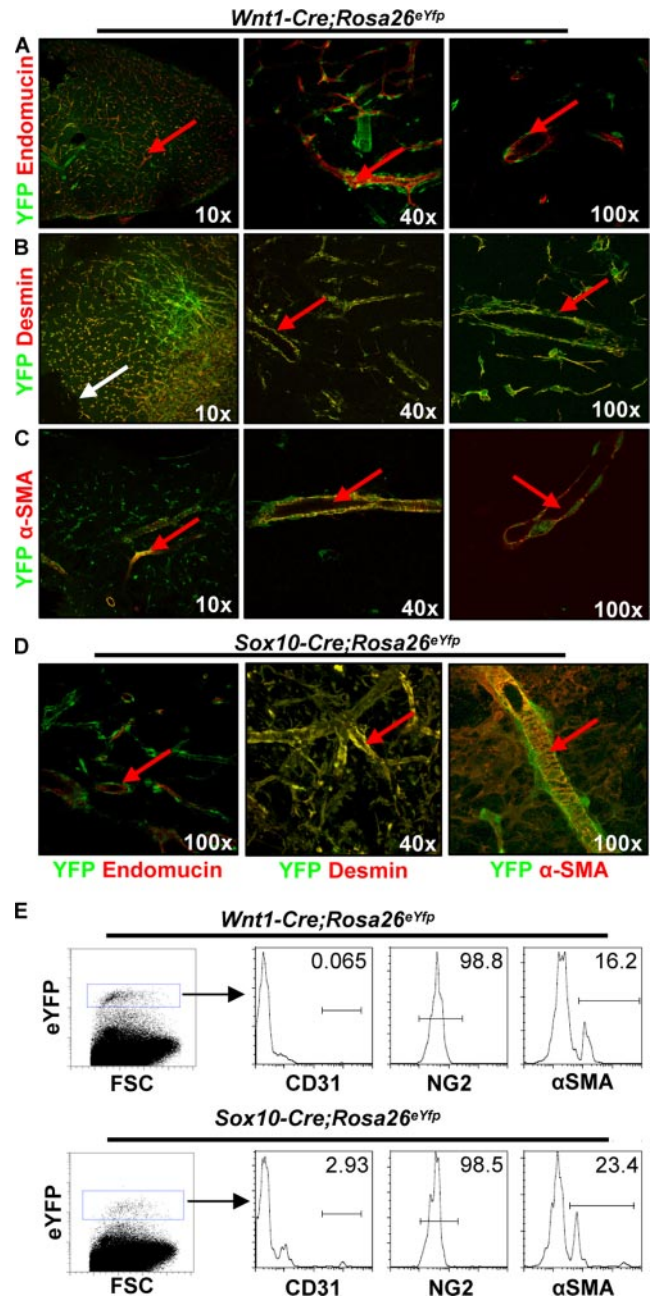
Mutations that knockout the function of PDGFR- $\alpha$  often result in hypoplastic but functional thymi; however, the role of PDGFR- $\beta$  signaling in the thymus is unknown. It has been shown that PDGFR- $\beta$  is required for recruitment of pericytes to the walls of blood vessels. Indeed, embryos deficient for this receptor do not survive beyond birth due to sudden microvascular hemorrhaging and edema formation (9, 26). We herein show that in both the embryonic and 3-mo-old thymus, NC-derived cells are PDGFR- $\beta^+$  (Fig. 4A), suggesting a mechanism by which NC-derived cells are recruited to the blood vasculature. However, by examining markers specific for pericytes and endothelial cells in E15.5 and E18.5 PDGFR- $\beta$ -deficient embryos, we show that in the absence of this signaling receptor the thymic blood vasculature appears to have developed normally (Fig. 4D).

#### NC-derived cells are maintained in the thymus into adulthood

It has been suggested that beyond E17.5 in thymus organogenesis, NC-derived mesenchymal cells are lost or replaced. However, as shown in Fig. 2C, NC-derived cells are still detected in the 3-mo-old organ. To determine the character of the persisting NC-derived cells in the adult thymus, we examined the expression of markers for blood vessel endothelium, pericytes, and smooth muscle cells (as described above in Fig. 3) at 3 mo of age. We show that eYFP $^+$  cells still surround endomucin-expressing endothelial cells (Fig. 5A), similar to the pattern observed in the embryonic thymus (Fig. 3). Because eYFP $^+$  cells appear to surround vessel structures, we assessed the expression of markers associated with pericytes (desmin) and smooth muscle cells ( $\alpha$ -SMA). We show that some vessel-associated eYFP $^+$  cells also express desmin and, in separate stainings,  $\alpha$ -SMA (Fig. 5, B and C). Identical patterns of expression were seen for endomucin, desmin, and  $\alpha$ -SMA using another mouse strain (*Sox10-Cre;Rosa26<sup>eYfp</sup>*) that also expresses Cre in NC-derived cells and thus marks all NC derivatives with eYFP (Fig. 5D). Furthermore, flow cytometric analyses of *Wnt1-Cre;Rosa26<sup>eYfp</sup>* and *Sox10-Cre;Rosa26<sup>eYfp</sup>* thymi show that there is similar expression of the endothelial marker CD31 (0.065 and 2.93%), NG2 (98.8 and 98.5%), and  $\alpha$ -SMA (16.2 and 23.4%, respectively) in these two reporter lines (Fig. 5E).

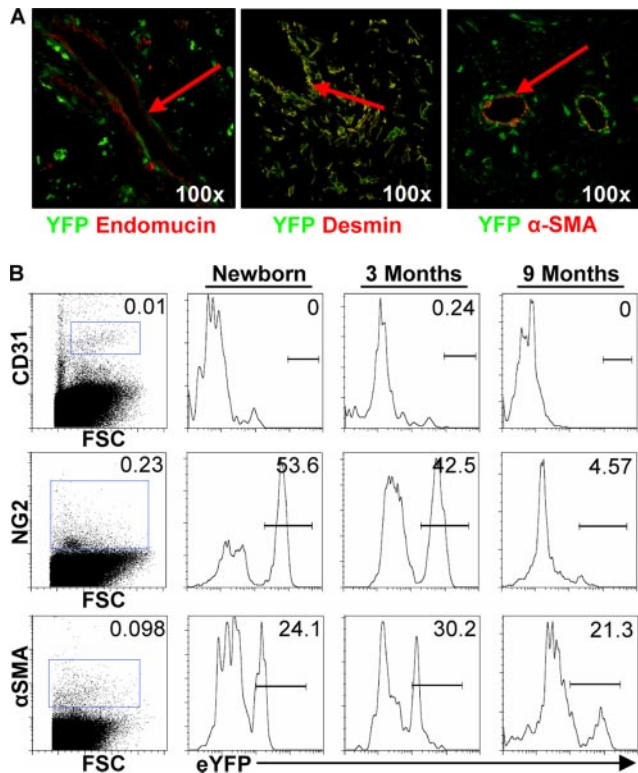
#### Age-related changes to the NCC contribution to the thymus

As the mouse ages, the perivascular space between the walls of the blood vessels and the surrounding cells in the thymus increases, and it is infiltrated with adipose tissue and lymphoid cells (27). This is correlated with a reduction in the export of new T cells into the periphery. To assess whether age-related atrophy is associated with a reduction in NC-derived cells, we have analyzed thymi from aged mice by both immunohistochemical stainings and flow cytometry. We show that at 9–10 mo of age, eYFP $^+$  cells are still present within the thymus and continue to surround blood vessel structures, but there appears to be a reduction in the number that coexpress desmin and  $\alpha$ -SMA (Fig. 6A). To confirm a decrease in the contribution of eYFP cells to the perivascular cell population during thymus organogenesis and aging, we analyzed thymi from mice at 9–10 mo of age by flow cytometry. We herein analyzed the percentage of CD31 $^+$ , NG2 $^+$ , and  $\alpha$ -SMA $^+$  cells that express eYFP. We show that the percentage of NG2 $^+$  cells that express eYFP decreases from 53.6% in the newborn thymus, to 42.5% at 3 mo of age, and to 4.57% by 9 mo of age. At birth the population of  $\alpha$ -SMA $^+$  cells that expresses eYFP is 24.1% of the total thymus, but there is an increase to 30.2% by 3 mo of age, indicating a differentiation or proliferation of existing eYFP $^+$  cells in this compartment. Similar to the results seen by confocal microscopy in Fig. 6A, there is a decrease in  $\alpha$ -SMA cells that express eYFP at 9 mo of age (21.3%) (Fig. 6B).



**FIGURE 5.** Differentiation of eYFP $^+$  cells in the adult thymus. A–C, Thymi from 3-mo-old *Wnt1-Cre;Rosa26<sup>eYfp</sup>* mice were dissected, processed as previously described, and stained with Abs against eYFP (green) and (A) endomucin (red), (B) desmin (red), and (C)  $\alpha$ -SMA (red), and analyzed by confocal microscopy. White arrows point to capsule of thymic lobe; red arrow, vessels. D, Thymi from 3-mo-old *Sox10-Cre;Rosa26<sup>eYfp</sup>* mice were dissected, processed as previously described, and stained with Abs against eYFP (green), endomucin (red, left), and  $\alpha$ -SMA (red, right). White arrows point to capsule of thymic lobe; red arrow, vessels. E, Thymi from 12-wk-old *Wnt1-Cre;Rosa26<sup>eYfp</sup>* (top) and 6-wk-old *Sox10-Cre;Rosa26<sup>eYfp</sup>* (bottom) mice were digested with collagenase, stained with markers recognizing endothelial cells (CD31), pericytes (NG2), and smooth muscle cells ( $\alpha$ -SMA), and analyzed by flow cytometry. Cells were gated on eYFP $^+$  expression, and the percentages of CD31 $^+$ , NG2 $^+$ , and  $\alpha$ -SMA $^+$  cells were calculated.

Taken together, these results show that NC-derived cells, which populate the thymus during organogenesis, persist in the adult thymus, having differentiated into at least two different cell types that form part of the same vascular supporting network. However, as



**FIGURE 6.** eYFP<sup>+</sup> cells within the aged thymus. **A**, Thymi from *Wnt1-Cre;Rosa26<sup>eYfp</sup>* mice at 10 mo of age were dissected and processed for confocal microscopy as previously described and stained with Abs recognizing eYFP (green) and endomucin (left), desmin (middle), and  $\alpha$ -SMA (right). Red arrows point to vessels. **B**, Thymi from *Wnt1-Cre;Rosa26<sup>eYfp</sup>* mice at birth, 3 mo of age, and 9 mo of age were collagenase digested and stained with Abs recognizing endothelial cells (CD31), pericytes (NG2), and smooth muscle cells ( $\alpha$ -SMA). The resulting single-cell suspension was first gated on CD31<sup>+</sup>, NG2<sup>+</sup>, and  $\alpha$ -SMA-expressing cells, and then the percentages of eYFP<sup>+</sup> cells within these populations were determined. Analyses of different time points were not conducted on the same day, and thus different gates are used to analyze the percentage of eYFP<sup>+</sup> cells from the gated populations.

the thymus atrophies with age, there is a reduction in the number of NC-derived cells which are associated with the perivascular network.

## Discussion

NC-derived cells are required for normal development of the thymus during the early stages of development. However, previous studies have suggested that NC-derived cells are reduced, or even replaced, by non-NC-derived mesenchymal cells toward later stages of organogenesis (E17.5) (17, 18). This is consistent with the view that once epithelial cells are functionally competent and the blood vasculature is complete, NC-derived cells are no longer required and they disappear or are replaced. Contrary to these results, we herein show that NC-derived cells populate the thymic rudiment, remain there throughout gestation, and persist in the adult thymus up to 9–10 mo of age (Fig. 1). Using Abs specific for pericytes and vascular smooth muscle cells, we show that NCCs differentiate into perivascular cells during thymus development (Fig. 3). In the aged mouse, the thymus atrophies and there is an alteration in the structure of the perivascular space by infiltration with adipose tissue. This change is associated with a gradual replacement in the perivascular component by cells of other mesenchymal origin (Fig. 6).

In 1975, Le Douarin and Jotereau (14) observed that the outer layer of the thymic capsule and connective tissues surrounding the blood vessels within the embryonic organ were derived from the neural crest. Using a transgenic reporter mouse line whereby LacZ expression is under the control of the *Wnt1* promoter, more recent studies have followed the contribution of NC-derived cells in the thymus from early organogenesis to birth, and they show that beyond E17.5, NC-derived cells are rarely detected (17, 18). However, using eYFP as a reporter of *Wnt1* expression, our data show that NC-derived cells do persist in the thymus beyond birth and remain there up to 9–10 mo of age. This apparent difference in expression of LacZ and eYFP, despite being under the control of the same promoter, may result from high CpG content in the *LacZ* gene and thus provide a predisposition to methylation-induced silencing (28). We herein show that in two independent mouse lines (*Wnt1-Cre* and *Sox10-Cre*), with each expressing Cre in a NCC pattern, eYFP<sup>+</sup> cells are detected in the 3-mo-old thymus with identical distribution (around vessels) and characteristics (expressing desmin/NG2 and  $\alpha$ -SMA). This makes it unlikely that eYFP expression is due to identical ectopic activation of the *Cre* gene.

In this report we describe different stages in blood vessel network development during fetal thymic differentiation. By E13.5, CD31<sup>+</sup> endothelial cells are scattered within the thymic rudiment. At this stage eYFP<sup>+</sup> cells form a network throughout the organ that is reminiscent of capillaries and that expresses the pericyte marker, desmin. Emergence of endothelial cells appears to coincide with NCC differentiation into perivascular cells, followed by mature blood vessel formation. However, the exact order of NCC and endothelial cell appearance in the thymic rudiment remains unclear. It is possible that endothelial precursor cells developing within the thymus attract NCCs into the rudiment and, subsequently, induce their differentiation into perivascular cells. It is equally possible that NC-derived cells enter the rudiment first and influence the migration of endothelial cells and the development of vascular networks, which in turn induce differentiation of NCCs into perivascular cells.

At E15.5, most NC-derived cells express PDGFR- $\beta$ , and many also express PDGFR- $\alpha$ . Herein, we show that in the adult thymus at 3 mo of age, NC-derived cells present at the capsule still express both mesenchymal markers PDGFR- $\alpha$  and PDGFR- $\beta$ . Furthermore, while NC-derived cells within the parenchyma down-regulate the expression of PDGFR- $\alpha$ , they maintain PDGFR- $\beta$  expression (Fig. 4). This change in pattern of expression appears to coincide with the migration of NCCs into the thymus during embryogenesis and their differentiation into perivascular cells, suggesting a switch from their role in supporting epithelial cell proliferation (29) to a role in blood vasculature development. A similar down-regulation of PDGFR- $\alpha$  has also been observed during pericyte differentiation in the brain, indicating similarities in differentiation of NC-derived cells into perivascular cells both in the thymus and in the brain (30).

In mice deficient for PDGFR- $\alpha$ , a small number of thymi are hypoplastic due to a lack in epithelial cell proliferation, but they are functionally competent (31, 32). The ligand for this receptor, PDGF-B, is produced by endothelial cells, resulting in the recruitment of pericytes to the walls of blood vessels. In the absence of either PDGFR- $\beta$  or PDGF-B there is a reduction in the number of perivascular cells associated with vessels, and there is a defect in endothelial cell proliferation. The function of PDGFR- $\beta$  in thymus development has not previously been characterized. We herein show that in the thymus of PDGFR- $\beta^{-/-}$  embryos, thymic blood vascular development and perivascular cell recruitment to vessels appear normal, indicating that other molecules may be involved.



In summary, we have characterized the contribution of NC-derived cells in the embryonic and adult thymus (up to 9–10 mo of age) and their involvement in blood vessel formation. We showed that NC-derived cells enter the thymus before E13.5, are present throughout gestation, and remain associated with the adult organ. These cells differentiate into perivascular cells, which may contribute to a postulated thymus-blood barrier (33, 34), and may also provide structural support to vessels and regulate endothelial cell function. Thus, NC-derived cells are not only important for early thymus organogenesis, but they may also have an important role in blood vessel development and the function of the postnatal thymus.

## Acknowledgments

We thank A. Rae, G. Preece, and C. Atkins for expert assistance in flow cytometry; T. Norton, K. Williams, and H. Boyes for assistance with animal work; and S. Pagakis, K. Sullivan, Y. Gu, and D. Zhu for assistance with confocal microscopy. We also thank Nicoletta Kassaris for Sox10-Cre and Naoko Kogata for PDGFR- $\beta^{-/-}$  mice.

## Disclosures

The authors have no financial conflicts of interest.

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